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Pharmacological and histochemical evidence for P2X receptors in human umbilical vessels

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Abstract

The presence of P2X purinoceptors in human umbilical vessels were studied with organ bath recording, radioligand binding assays, autoradiography, and immunohistochemistry. In isolated umbilical arteries and veins from normal term pregnancy, both ATP and α,β-methylene ATP caused concentration-dependent contractions. ATP-induced responses were blocked by desensitisation with α,β-methylene ATP. However, both the ATP- and α,β-methylene ATP-induced responses were not antagonised by suramin. No significant difference in responses was observed in the vessels with or without endothelial cells. Radioligand binding assays using [^{3}H]α,β-methylene ATP showed the presence of a population of high-affinity binding sites in both the arteries and veins. The K_d values of the binding sites were 2.77 ± 1.10 nM for the arteries, and 3.23 ± 1.22 nM for the veins. The maximum binding site densities were 634 ± 237 and 947 ± 308 fmol/mg protein for the arteries and the veins, respectively. Autoradiographic localisation with [^{3}H]α,β-methylene ATP demonstrated that the specific binding sites were only distributed over the smooth muscle cells of the vessels. Immunohistochemical studies with specific polyclonal antibodies against $P2X_{1-6}$ receptors showed that positive immunostaining was also restricted to smooth muscle cells. Antibodies against $P2X_1$ receptors produced the strongest signals, while antibodies against the other five P2X subtypes produced much weaker signals. The results in the present study indicate the existence of P2X purinoceptors in the smooth muscle of human umbilical vessels. Their physiological functions remain to be studied.

Keywords: P2X receptor; Purinoceptor; ATP; α,β-Methylene ATP; Suramin; Umbilical artery, human; Blood vessel

1. Introduction

Human term placenta and umbilical cord are non-innervated. However, many bioactive agents including adenosine and ATP can induce contractions or dilatation of placental vasculature. Systemic administration of adenosine to normoxic fetal lambs has been reported to induce an immediate vasoconstriction followed by vasodilatation (Reid et al., 1990). On isolated human chorionic arteries and veins, the relaxation induced by adenosine was suggested to be mediated via both the A_1 and A_2 receptors (Reviriego et al., 1990). In perfused lobules of human placenta, ATP can also induce a biphasic response in perfusion pressure, with an initial transient increase followed by a decrease (Read et al., 1993), which indicates the existence of both P2X and P2Y receptors in the human placental vasculature. The P2Y receptors were believed to be present on the endothelial cells (Carter et al., 1988, 1990; Read et al., 1993; Pirotton et al., 1996; Ralevic et al., 1997). Upon activation, the receptors can mediate the release of prostacyclin (Carter et al., 1988, 1990) and nitric oxide (Read et al., 1993; Ralevic et al., 1997), which in turn will induce vasodilatation. Several subtypes of cloned P2Y receptors including $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ have been reported to be present in the human placenta (Communi et al., 1995, 1996; Ayyanathan et al., 1996; Leon et al., 1996; Southey et al., 1996), and may play important roles in the regulation of local blood circulation.

Compared with the reports on P2Y receptors in the placenta and umbilical vessels, the reports on P2X receptors in these tissues are scarce. Two recent reports suggested that P2X receptors present in human placental blood vessels were different from those in arteries of other tissues (Dobronyi et al., 1997; Ralevic et al., 1997). We have found that the main subtype of P2X receptors in vascular smooth muscle cells is the $P2X_1$ (unpublished data). In another report, the rat $P2X_1$ mRNA transcripts

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were identified in the umbilical vessels with in situ hybridisation (Valera et al., 1994). In order to provide further evidence on the existence of P2X receptors in umbilical vessels, we examined the P2X receptors in human umbilical arteries and veins with in vitro organ bath recording, radioligand binding, autoradiographic localisation, and immunohistochemistry.

2. Materials and methods

2.1. Pharmacology

Human term umbilical cord was obtained from uncomplicated pregnancies after vaginal delivery. Segments of arteries and veins (about 5 mm in length) were dissected out and mounted horizontally in 5-ml organ baths containing modified Kreb's solution of the following composition: NaCl 118 mM, KCl 4.7 mM, NaH_2PO_4 1.0 mM, $NaHCO_3$ 25 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, glucose 11.1 mM, bovine serum albumin 2.5 mg/l, and bacitracin 1000 units/ml. The solution was continually aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. Isometric tension was recorded with a Grass FTO3C transducer and displayed on a Grass ink-writing polygraph (model 79). An initial load of 2 g was applied and the tissues were allowed to equilibrate for 2 h, resulting in an approximate tension of 1.5 g. The endothelium of some preparations was removed by gentle rubbing of the intimal surface with a pair of serrated forceps.

KCl (120 mM) was initially used to induce vasoconstriction. The vascular responses to the purine compounds were expressed as a percentage of the KCl-induced contraction. Concentration–response curves to ATP and α,β-methylene ATP (α,β-MeATP) were constructed in single concentrations to avoid desensitisation.

2.2. Radioligand binding assay

The umbilical arteries and veins were dissected out as described above. They were minced and homogenised in cold 50 mM Tris–HCl buffer containing 1 mM EGTA, 1 mM benzamidine hydrochloride, 0.1 mM phenylmethylsulphonyl fluoride, 0.01% bacitracin, and 0.002% soybean trypsin inhibitors (Buffer A, pH 7.4). The homogenate was centrifuged at $170 \times g$ for 5 min. The supernatant was passed through double layers of nylon mesh and centrifuged at $105{,}000 \times g$ at 4°C for 1 h. The pellet was suspended in Buffer A. Protein concentration was determined by the method of Lowry et al. (1951).

For saturation analysis, the membrane preparations (about 10 μg protein per tube) were incubated with a series of concentrations of [^{3}H]α,β-MeATP at 4°C for 2 h. Non-specific binding was determined in the presence of 100 μM β,γ-methylene ATP (β,γ-MeATP). The reaction was terminated by vacuum filtration of the reaction mixture through Whatman GF/B filters which had been presoaked in 20 mM disodium pyrophosphate in order to reduce non-specific binding to the filters. The filters were washed with two aliquots of 5 ml ice-cold 50 mM Tris–HCl buffer. The radioactivity entrapped in the filters was measured in a scintillation counter. Each experiment was carried out in triplicate.

2.3. Autoradiography

Small segments of umbilical cords were embedded and frozen in precooled isopentane. Cryostat sections (14 μm in thickness) were cut and thaw-mounted onto gelatine-coated slides. Slide-mounted sections were preincubated in Buffer A at 30°C for 10 min to remove endogenous ligands and embedding matrix. They were then transferred to Buffer A containing 10 nM [^{3}H]α,β-MeATP and incubated at 30°C for 15 min. Non-specific binding was determined in the presence of 100 μM β,γ-MeATP. At the end of the incubation, the sections were washed in ice-cold 50 mM Tris–HCl buffer and rinsed in ice-cold distilled water. The time and temperature for the incubation and the wash had been optimised in preliminary experiments. The autoradiograms were produced by the method of Young and Kuhar (1979): an emulsion-coated coverslip was attached to one end of each slide with adhesive. The assemblies were exposed for 2 weeks at 4°C. The emulsion was developed and fixed, and the sections were stained with 0.5% toluidine blue.

2.4. Immunohistochemistry

Umbilical sections were cut as described above. Sections were fixed in 2% paraformaldehyde and 0.2% picric acid. Sections were first blocked with 10% skimmed milk, and then with blocking solutions from Vector Laboratories. Rabbit polyclonal antibodies against $P2X_1$ receptors were diluted with 5% skimmed milk and applied to the sections overnight at 4°C. The sections were washed in phosphate-buffered saline. Biotinylated donkey anti-rabbit immunoglobulin antibodies were diluted in 5% skimmed milk and applied to the sections for 1 h at room temperature. The avidin–biotin signal amplification complex (Vectastain Elite ABC kit) was applied for 30 min at room temperature. Finally, a solution containing 0.5 mg/ml, diaminobenzidine, 0.04% nickel amino sulphate, 0.2% β-D-glucose, 0.004% amino nitrate, 1.2 units/ml oxidase glucose in 0.01 M phosphate-buffered saline were applied until reaction product was visualised. For controls, pre-immune rabbit sera were applied or the primary antibodies were omitted.

2.5. Drugs

The [^{3}H]α,β-MeATP was synthesised by Amersham International (Amersham, UK). Specific activity was 19.1 Ci/mmol with the chemical purity of 98–99%. Suramin was purchased from Research Biochemicals (Natick, USA).

The biotinylated donkey anti-rabbit immunoglobulin antibody was purchased from Amersham International (Amersham, UK). Polyclonal antibodies against $P2X_{1-6}$ receptors were produced by Roche Bioscience (Palo Alto, USA). Blocking kit and Vectastain Elite ABC kit were purchased from Vector Laboratories (Peterborough, UK). All the other chemicals were purchased from Sigma (Poole, UK).

2.6. Preparation and specificity of the P2X antibodies

The immunogens used were synthetic peptides corresponding to the carboxy termini of the cloned rat P2X receptors, covalently linked to Keyhole Limpet Haemocyanin. The peptide sequences are as follows: $P2X_1$, amino acid 385–399, ATSSTLGLQENMRTS; $P2X_2$, amino acid 458–472, QQDSTSTDPKGLAQL; $P2X_3$, amino acid 383–397, VEKQSTDSGAYSIGH; $P2X_4$, amino acid 374–388, YVEDYEQGLSGEMNQ; $P2X_5$, amino acid 437–451, RENAIVNVKQSQILH; $P2X_6$, amino acid 357–371, EAGFYWRTKYEEARA. Polyclonal antibodies were raised in New Zealand rabbits by multiple monthly injections. The specificity of the antisera were verified by immunoblotting with membrane preparations from native tissue sources (rat vas deferens for $P2X_1$ and rat dorsal root ganglion for $P2X_3$) and/or Chinese hamster ovary cell line (CHO K1 cells) expressing the cloned $P2X_{1-6}$ receptors. The IgG fractions were isolated from the immune sera and the pre-immune controls using chromatography on DEAE Affi-Gel blue gel (Bio-Rad, Hemel Hempstead, UK).

2.7. Data analysis

All results are expressed as means ± S.E.M., with *n* referring to the number of umbilical vessels. Radioligand binding assay data were analysed with the computer programme, RADLIG 4.0 (Biosoft, Cambridge, UK).

3. Results

3.1. Pharmacology

At basal tone, ATP caused concentration-dependent transitory contractions of the human umbilical arteries

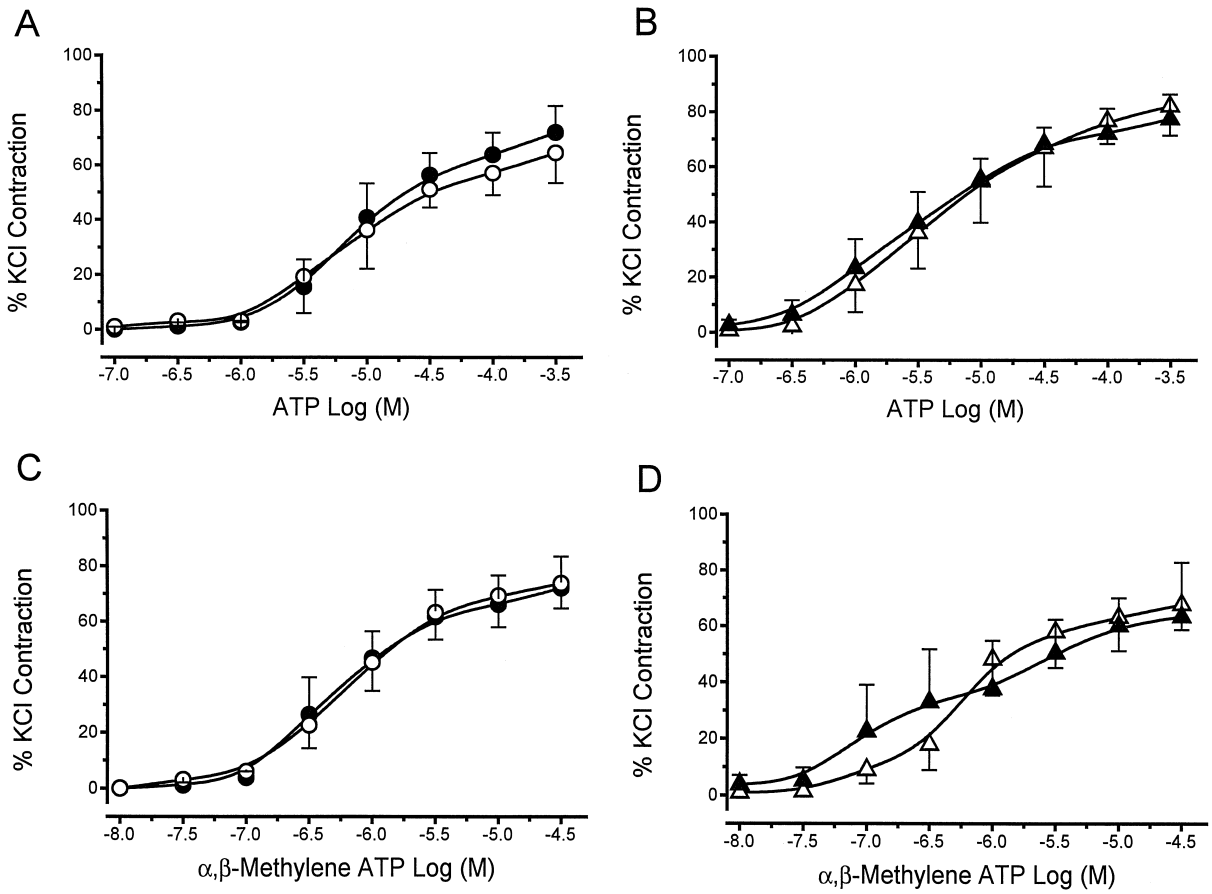

Fig. 1. Concentration–response curves of human umbilical vessels to ATP and α,β-methylene ATP. (A) Effects of ATP on umbilical artery in the absence (○) and presence (●) of an intact endothelium ($n = 6$). (B) Effects of ATP on umbilical vein in the absence (Δ) and presence (▲) of an intact endothelium ($n = 6$). (C) Effects of α,β-methylene ATP on umbilical artery in the absence (○) and presence (●) of an intact endothelium ($n = 6$). (D) Effects of α,β-methylene ATP on umbilical vein in the absence (Δ) and presence (▲) of an intact endothelium ($n = 6$). Results (mean ± S.E.M.) are expressed as percentage of responses induced by 120 mM KCl.

(Fig. 1A) and veins (Fig. 1B) in the absence and presence of an intact endothelium. The α,β-MeATP also produced similar concentration–response curves in umbilical arteries (Fig. 1C) and veins (Fig. 1D). The EC_{50} values for individual concentration–response curves were not calculated because in some preparations, the response did not reach maximum. However, by visual comparison of the concentration–response curves, it appeared that α,β-MeATP was more potent than ATP. Contractions to ATP were abolished by desensitisation with α,β-MeATP (repeated application of 3 μM α,β-MeATP). Suramin at concentration up to 1 mM did not inhibit the ATP or α,β-MeATP-induced contraction. On the contrary, suramin itself caused vasoconstriction in a number of preparations. Indomethacin (50 μg/ml) also showed no inhibitory effect on the responses to ATP and α,β-MeATP.

3.2. Radioligand binding

The binding of [^{3}H]α,β-MeATP to the membrane preparations of human umbilical vessels was saturable (Fig. 2A). Scatchard analysis revealed the existence of high-affinity [^{3}H]α,β-MeATP binding sites (Fig. 2B). The maximum density of the binding sites was 634 ± 237 fmol/mg protein ($n = 4$) for the umbilical artery and 947 ± 308 fmol/mg protein ($n = 3$) for the umbilical vein, with apparent dissociation constants (K_d) of 2.77 ± 1.10 nM and 3.23 ± 1.22 nM, respectively. Both the binding site densities and the K_d values were not significantly different between the artery and vein preparations.

3.3. Autoradiography

Specific binding sites of [^{3}H]α,β-MeATP were found only over the smooth muscle cells of both the umbilical artery and vein (Fig. 3). In the presence an excess concen-

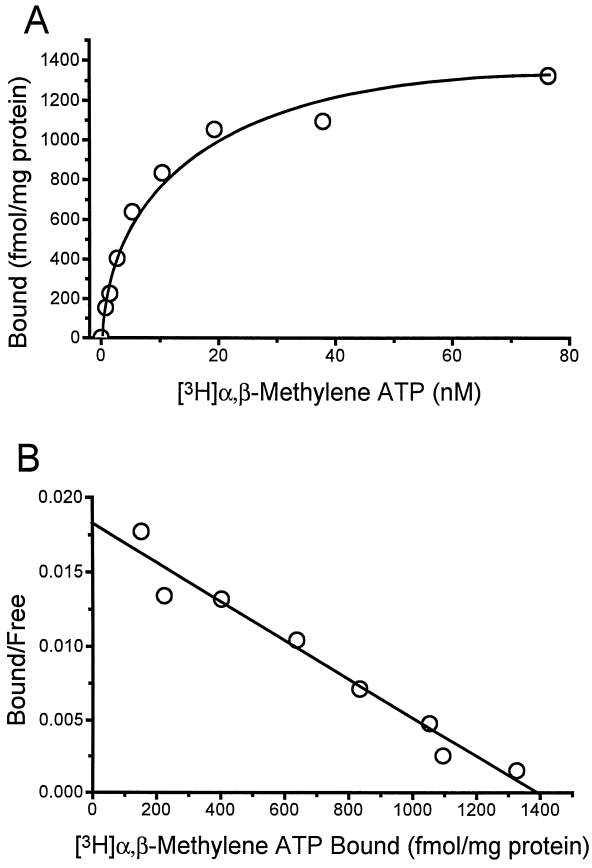

Fig. 2. The [^{3}H]α,β-methylene ATP binding to the membrane preparations of human umbilical arteries and veins. (A) A representative of saturation curves. (B) A Scatchard plot of the same group of data as (A).

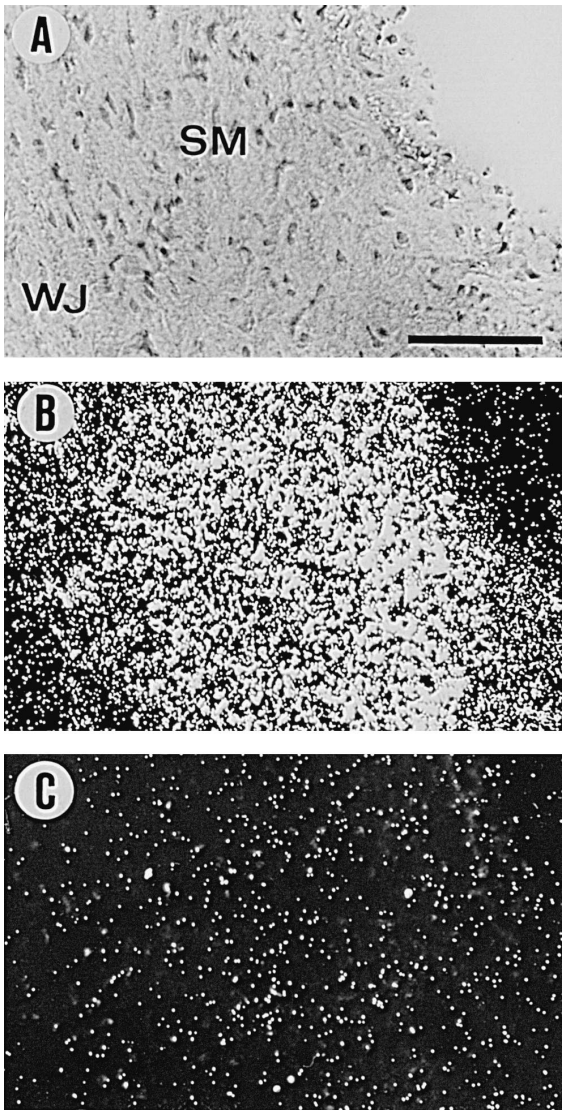

Fig. 3. Autoradiographs of human umbilical artery demonstrating specific [^{3}H]α,β-methylene ATP binding sites. (A) Bright-field views of the human umbilical artery stained with 0.5% toluidine blue. (B) Dark-field view of (A), showing the overall distribution of [^{3}H]α,β-methylene ATP binding sites in the section. (C) Dark-field views of the adjacent sections of (A), showing the non-specific binding sites of [^{3}H]α,β-methylene ATP in the section. SM: smooth muscle. WJ: Wharton's jelly. Calibration bar: 100 μm.

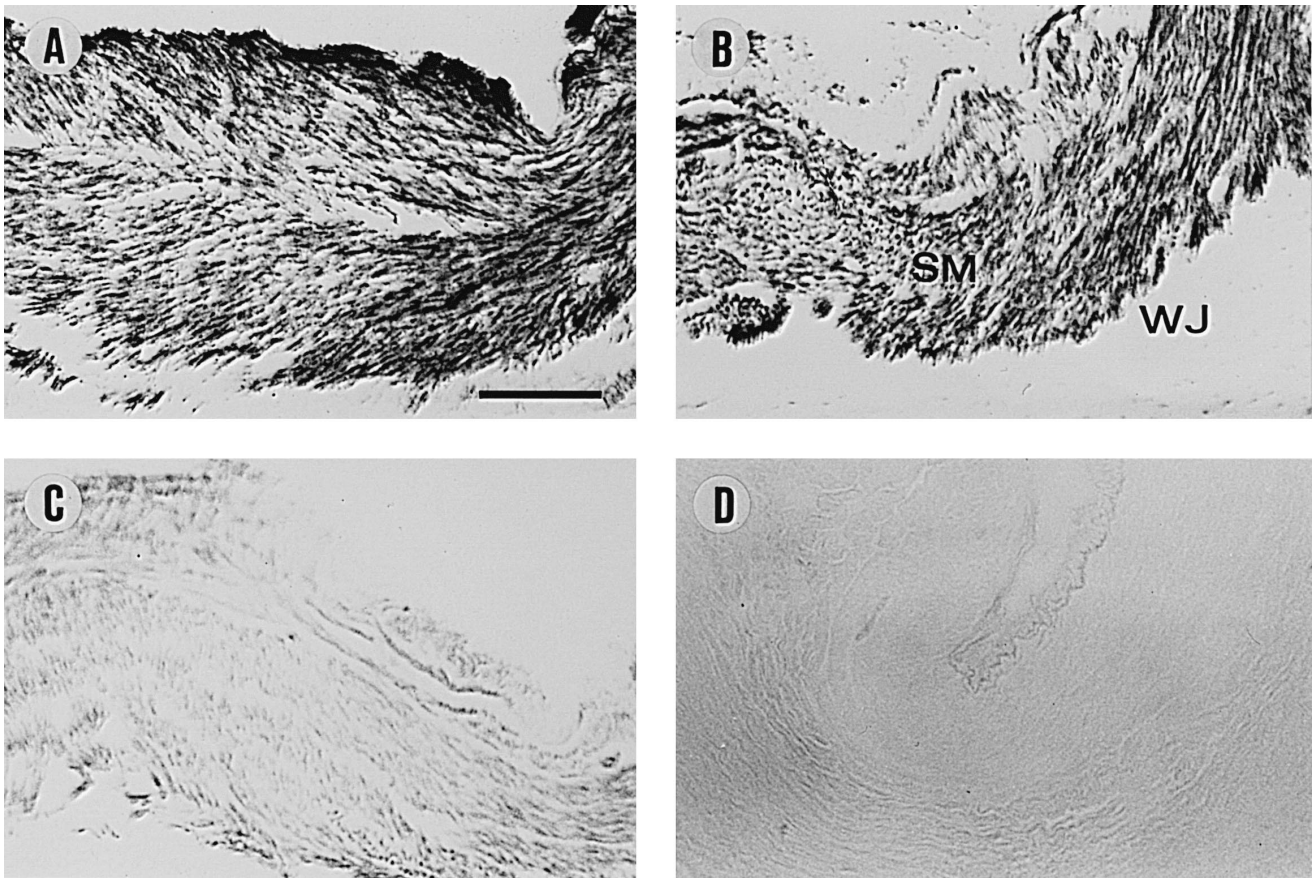

Fig. 4. Immunostaining of human umbilical artery and vein with specific polyclonal antibodies against P2X receptors. (A) Umbilical artery stained with $P2X_1$ receptor antibodies. (B) Umbilical vein stained with $P2X_1$ receptor antibodies. (C) Umbilical vein stained with $P2X_2$ receptor antibodies. (D) Umbilical vein with the primary antibody omitted. SM: smooth muscle. WJ: Wharton's jelly. Calibration bar: 100 μm.

tration of β,γ-MeATP, the [^{3}H]α,β-MeATP binding site density was significantly reduced (Fig. 3C).

3.4. Immunohistochemistry

As with the distribution of specific [^{3}H]α,β-MeATP binding sites revealed by autoradiography, immunohistochemistry showed that positive staining with specific P2X receptor antibodies was also limited to the smooth muscle of the umbilical arteries and veins (Fig. 4A,B,C). When pre-immune rabbit sera were used or when the primary antibody was omitted, no detectable positive staining was observed in the sections (Fig. 4D). Antibodies against $P2X_1$ receptor produced the strongest signal whereas antibodies against other five subtypes produced weak signals which were more or less of the same intensity (Fig. 4C).

4. Discussion

Both ATP and α,β-MeATP induced concentration-dependent contractions in human umbilical arteries and veins. The ATP-induced response was blocked by desensitisation with α,β-MeATP, which is a typical feature for this type of P2X receptors (Burnstock and Kennedy, 1985). The ATP- and α,β-MeATP-induced contractions were transitory because of the rapid desensitisation of P2X receptors which are ATP-gated cation channels. Electrophysiological studies on the cloned P2X receptors have found that mainly the $P2X_1$ and $P2X_3$ subtypes are desensitised by α,β-MeATP (Valera et al., 1994; Chen et al., 1995). In the present study, we believe that the contraction of human umbilical vessels was predominantly mediated by $P2X_1$ receptors or a heteromultimer of $P2X_1$ with other subtypes as was shown in the immunohistochemical experiments.

Suramin has been used widely as an antagonist for P2X receptors (Voogd et al., 1993). It is also used to discriminate the cloned P2X subtypes (Burnstock and King, 1996; North and Barnard, 1997). Among the seven cloned P2X receptors, $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$ are sensitive to suramin, while $P2X_4$, $P2X_6$ and $P2X_7$ are insensitive. In the present study, the P2X receptors could be desensitised by α,β-MeATP while insensitive to suramin. In another study on perfused human placental cotyledons, ATP- and α,β-MeATP-induced vasoconstriction was resistant to desensitisation by α,β-MeATP and not affected by the P2X receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (Ralevic et al., 1997). Interestingly,

in human chorionic surface artery, it has been reported that the α,β-MeATP did not desensitise the P2X receptor, whereas it could be inhibited by suramin (Dobronyi et al., 1997). A simple explanation cannot be given to the three different kinds of P2X receptor pharmacological profiles in human placental vasculature. It is possible the functional P2X receptors are in the form of a heteromultimer of $P2X_1$ and other P2X subtypes like the suramin-insensitive subtype. The existence of heteromultimers of P2X receptors in other blood vessels has been implicated (Nori et al., 1998). The occasional vasoconstriction observed in some vessels after the application of suramin is somewhat unusual; however, a hyperpolarisation was observed in the guinea pig superior cervical ganglia on application of suramin at equivalent concentrations (Reekie and Burnstock, 1994). The underlying mechanism for such phenomenon is unclear at the moment.

In the present study, indomethacin also did not inhibit the ATP- or α,β-MeATP-induced responses. This contrasts with the report from Fiscus and Dyer (1982) in which ATP-induced contractions in human umbilical artery were completely inhibited by indomethacin, leading the authors to suggest that ATP-induced contractions were mediated by prostaglandin-like substances. Such discrepancy cannot currently be explained. Since P2X receptors are fast ligand-gated cation channels, activation of these receptors can lead to depolarisation and smooth muscle contraction. The possibility that prostaglandins are involved in the P2X receptor-mediated vasoconstriction seems very small.

The removal of endothelial cells did not significantly affect the ATP- and α,β-MeATP-induced responses, indicating that P2X receptors are localised on the smooth muscle cells of the vessels. The P2Y receptors were reported to be present on the endothelial cells of umbilical arteries and veins (Carter et al., 1988, 1990; Hawley et al., 1995; Pirotton et al., 1996). The activation of P2Y receptors on endothelial cells may lead to the release of prostacyclin and endothelial-derived relaxing factors such as nitric oxide, which induce vasodilatation. However, such mechanism did not significantly affect the ATP-induced vasoconstriction at basal tone in the present study.

Unlike the binding sites in the rat urinary bladder and vas deferens where high- and low-affinity sites have been identified (Bo and Burnstock, 1990; Bo et al., 1992), radioligand binding assays demonstrated the existence of only the high-affinity [^{3}H]α,β-MeATP binding site in the human umbilical vessels. The densities of the binding sites were significantly lower than those in the urinary bladder and vas deferens, which may be because these tissues are under the control of purinergic neurotransmission. Autoradiography confirmed that [^{3}H]α,β-MeATP binding sites were located on the smooth muscle cells of the umbilical vessels.

The synthetic peptides corresponding to the C-termini of the cloned rat P2X receptors share no homology in amino acid sequence. Therefore, the polyclonal antibodies raised with these peptides did not show cross-reaction to other receptor subtypes. The homology between the rat and human P2X analogues were quite high; the difference in amino acid sequence ranges from zero ($P2X_1$) to three ($P2X_2$) amino acids out of the 15 C-terminal amino acids (the human $P2X_5$ analogue has not been reported). Because the sequence of the C-terminal peptide for $P2X_1$ receptor is shared by human and rat analogues (Valera et al., 1995), it is speculated that the polyclonal antibodies raised with this peptide can recognise both the human and rat $P2X_1$ receptors. On human umbilical vessels, $P2X_1$ receptor antibodies intensively stained the smooth muscle cells, matching the results obtained from autoradiography. However, all the other five P2X receptor antibodies also stained the smooth muscle cells weakly, possibly indicating the heteromultimerism of P2X receptors. If the functional P2X receptors do exist as heteromultimers, the atypical P2X receptor-mediated responses in the placental vasculature might be explained. However, the true composition of the native P2X receptors remains unclear at the moment.

5. Conclusion

The results from the in vitro organ bath recording, radioligand binding assays, autoradiography, and immunohistochemistry in the present study indicate the presence of P2X receptors in the smooth muscle cells of human umbilical arteries and veins. Their physiological functions and involvement in pathological mechanisms will need further investigation.

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